# METHOD AND KIT FOR ENHANCED DIFFERENTIAL DISPLAY

## BACKGROUND OF THE INVENTION

The field of the present invention relates to techniques for screening differences in gene expression between various cell types or between different stages of cell development.

In higher organisms, every cell expresses about 10–20% of the 100,000 possible different genes. Gene expression is involved in all life processes, such as development, aging and disease states. Thus, the analysis of which genes are expressed at any given time, and the identification of the expressed mRNAs, is of prime interest in molecular biology.

One such method for screening differences in gene expression is known as Differential Display. This method is described in Pardee et al., U.S. Pat. No. 5,262,311, hereby incorporated by reference. Differential Display involves amplifying partial cDNA sequences from subsets of mRNAs by reverse transcription and the polymerase chain reaction (PCR), then displaying these sequences on a sequencing gel.

In the Differential Display method, the primers which hybridize to the 3' end of the mRNA [the 3' primers] are selected to take advantage of the polyadenylate [polyA] tail present on most eukaryotic mRNAs to anchor the primers at the 3' end of the mRNA. Each 3' primer hybridizes to a portion of the polyA tail and additionally to 2 bases which are immediately 5' of the polyA tail. The 2 nucleotides of the 3' primer which are not complementary to the polyA tail are of the sequence VN, where V is deoxyadenylate ("dA"), deoxyguanylate ("dG"), or deoxycytidylate ("dC"), and N, the 3' terminal nucleotide, is dA, dG, dC, or deoxythymidylate ("dT"). By probability, each 3' primer Will recognize one-twelfth of the total mRNA population, since there are twelve different combinations of the two 3' bases, eliminating T as the base which hybridizes immediately 5' of the polyA tail. Such primers are used to reverse transcribe specific subpopulations of mRNAs.

A second set of primers, the 5' primers, is designed to randomly select a subset of the cDNAs generated using the 3' oligonucleotide primers. The 5' primers are of arbitrary base sequence. The cDNA sequences defined by these two primer sets are then amplified by PCR. The amplified products can then be displayed on a sequencing gel, and visualized by autoradiography. Using this method, comparisons can be made of the genes expressed by different cell types, or by cells in various stages of development or disease.

Differential Display uses short 9 to 14 base primers that require low temperature annealing throughout PCR amplification. Such low temperature annealing conditions result in a decline in the reproducibility of the method. As noted by the creators of Differential Display (Liang et al., Nucl. Acids Res. 21:3269–3275), "[a] troublesome aspect of the method is that the noise level of false positives, though a few percent, can be very appreciable relative to the truly different bands between cells."

## SUMMARY OF THE INVENTION

Applicant provides an improved method of Differential Display, named Enhanced Differential Display (EDD). EDD is designed as a technique for screening differences in gene expression between various cell types or between different stages of cell development. The technique is highly reproducible, leading to precise typing of the expressed genes in any given cell. EDD analysis permits the identification of novel genes involved in differentiation, aging, and disease, and enables direct comparisons of different cell types and disease states.



EDD uses the polymerase chain reaction (PCR) to amplify cDNA produced from a selected set of expressed mRNA sequences from particular cell types. The EDD method is similar to Differential Display, which also uses reverse transcriptase and PCR to identify differentially expressed genes. However, unlike Differential Display, which uses short 9 to 14 bases primers, EDD uses longer primers.

We have surprisingly found that by using longer primers, and/or an alteration in the annealing temperatures, the number of false positives can be significantly reduced. By "longer primers" it is meant that the primers are of at least 21 nucleotides in length, and can be up to 50 nucleotides in length. Most preferably, the oligonucleotide primers are between 22 and 30 nucleotides in length.

Thus, in a first aspect, the invention features an improved method for detecting differences in gene expression which comprises, first, contacting nucleic acid which comprises an mRNA sequence with a first oligonucleotide primer, wherein said first oligonucleotide primer has a hybridizing sequence sufficiently complementary to a region of said mRNA to hybridize therewith. Next, the first oligonucleotide primer is extended in an extension reaction using the mRNA as a template to give a first DNA primer extension product complementary to the mRNA. The first DNA primer extension product is then contacted with a second oligonucleotide primer, wherein the second oligonucleotide primer has a hybridizing sequence sufficiently complementary to the first DNA primer extension product to hybridize therewith. The second oligonucleotide primer is then extended in an extension reaction using the first DNA primer extension product as a template to give a second DNA primer extension product complementary to the first DNA primer extension product, and the first and second DNA primer extension products are amplified. The improvements of this method comprise one or more of the following: providing first and second oligonucleotide primers with a length of at least 21 oligonucleotides; the use of a two-step PCR amplification; and, not adding additional 3' oligonucleotide primers to the PCR amplification reaction mixture.

In a preferred embodiment, the PCR amplification is carried out in two steps. The first one to four cycles of PCR are carried out under non-stringent conditions. By "non-stringent" conditions it is meant that low annealing temperatures are used. Preferably, the annealing temperature used for the non-stringent conditions cycles is between 35° C. and 45° C. Most preferably, the annealing temperature used for the non-stringent conditions cycles is about 41° C. The next 16 to 20 cycles of amplification are carried out in stringent conditions. By "stringent" conditions it is meant that higher annealing temperatures are used. Preferably, the annealing temperature used for the stringent conditions cycles is between 55° C. and 70° C. Most preferably, the annealing temperature used for the stringent conditions cycles is about 60° C.–65° C. The buffer conditions used for both the stringent and non-stringent cycles are the same. An example of the annealing conditions used for both the stringent and nonstringent cycles is: 1 μl cDNA (3'primer carried over from cDNA), 2 μl 10x PCR buffer, 1.5 μl 0.1 mM dNTP, 1.25 μl 20 μM 5'primer, 1 μl 1 to 5 dilution of alpha-$^{32}$P dATP, 0.5 μl Taq polymerase, and 12.75 μl $dH_2O$.

"Cycle" refers to the process which results in the production of a copy of a target nucleic acid. A cycle includes a